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Application of an *In Vitro* Alveolus Model in Evaluating the Alveolar Response to Pressure-Induced Injury

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***Abstract*—**In an effort to understand the preliminary effects of aerodynamic stress on alveolar epithelial cells, we developed a multifluidic cell culture platform capable of supporting alveolar cultures at an air-liquid interface under constant air flow and exposure to varying pressure stimuli on the apical side while providing nourishment on the basolateral plane. Our current study involved utilizing the platform to study the effect of basement membrane coating and addition of dexamethasone on cellular response to pressure in A549 and H441 alveolar epithelial cells.

***Keywords*—**Aerodynamic stress, Air-liquid interface, Alveolar, Dexamethasone.

I. Introduction

ACUTE Lung Injury induced by mechanical ventilation is a frequent cause of aggravation or induction of pulmonary injury with high morbidity and mortality. Mechanical ventilation in conjunction with administration of concentrated oxygen form a vital part of therapy in treating premature born babies with underdeveloped lungs or existing congenital conditions and in adults with chronic obstructive pulmonary disease. However, these initial strategies have shown to lead to lung injury caused by hyperoxia [1], [2] and supra-physiological pulmonary pressure at the alveoli [2]. Although this has been studied predominantly in whole-animal models [2], [3], the underlying mechanisms for trauma are not very well understood. In this study we utilize a multi-fluidic *in vitro* model capable of exposing alveolar epithelial cells, cultured at an air-liquid interface, to aerodynamic stress to investigate cellular response in changes to the integrity of the gas exchange interface.

II. Materials and Methods

A. Cell Culture Chamber Design and Set Up

The cell culture device used in this study has the capability of exposing alveolar cells to varying aerodynamic stress. The design considerations of the culture device included separate side- and end- sample ports, detachable basal-viewing and top-access panels as depicted in Fig. 1. The culture device also overcomes a few shortcomings of conventional culture systems such as the ability to have a continuous low-flow medium replenishment on the basolateral surface while maintaining steady air exposure on the apical side of the epithelial culture, thus better mimicking their physiological microenvironment. It also allows for easy access to the culture wells facilitating top seeding of cells as well as apical sample collection. Since the medium flow rate is very low, effluent collection is an easier task with minimal dilution of contents for further testing. The side ports provide access to electrodes for the transepithelial electrical (TEER) measurements without disturbing the cellular layer or introducing air bubbles.

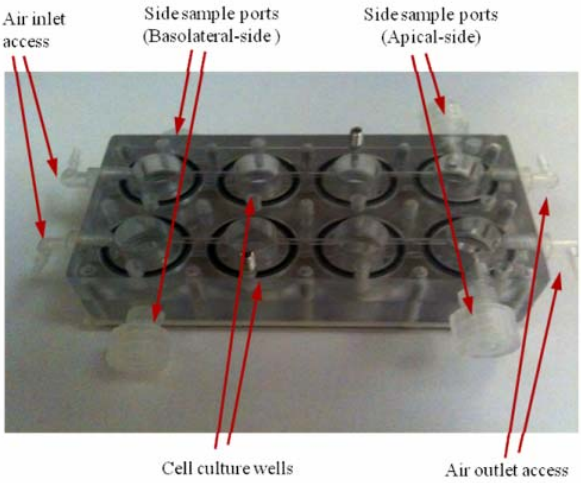

Fig. 1 Image of the cell culture device prototype highlighting the culture wells, side sample ports and end access ports. The top viewing panel covers the culture wells forming conduits for air flow

The culture device incorporates a suspended polyethylene terephthalate (PET) membrane with 0.3 µm pores for cell culture surface, sandwiched between two layers of clear polycarbonate that house the wells and conduits for air and medium supply. There are two parallel sets of four wells in series, allowing for high throughput testing under identical culture conditions.

The medium-side flow is a series connection starting with a

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medium reservoir followed by a variable speed ultra low flow pump (Cat.05949-29, Fisher Scientific), a bubble trap, the lower compartment of the culture device and an effluent collection tube, all connected using silastic tubing. Similarly the air-side connection is a simple water-trap circuit post-chamber with a low-flow air pump (knf lab pump, Cole-Parmer) at the inlet. The pressure in the chamber was monitored using a DP-Calc digital manometer (Cat. 05949-29, Cole-Parmer).

B. Cell Culture

Human alveolar epithelial cell lines, A549 (ATCC# CCL-185) and H441 (ATCC# HTB-174) cultured in F12k (Cat. 10-025-CV, Corning Cellgro) and RPMI 1640 (112-025-101, Quality Biological) respectively, supplemented with 10% FBS (Cat. 16140071, Invitrogen) and Penn-Strep (Cat. 120-095-721, Quality Biological) were used in this study. Cells were kept within passages 2-8 to avoid phenotype changes from prolonged culture. Cells were grown to 70% to 85% confluence in tissue culture flasks, before being harvested using 0.25% Trypsin EDTA (Cat. 25300-054, Gibco) for the experiments.

C. On-chip Cell Culture and Measurements

Epithelial cells were grown in the custom-built culture device, capable of exposing cells to aerodynamic stress, for 3 days prior to the introduction of the stimuli. Transepithelial electrical resistance (TEER), a measure of the alveolar membrane integrity, was measured every 24 h to register any changes to the cellular surface. Simultaneous fluorescent images were taken every 24 h to ascertain cell viability and composition.

For trials testing the effect of matrigel coating on alveolar cell response, each 1.2 cm diameter PET membrane was coated with 50 μL matrigel solution (Cat.E1270 Sigma-Aldrich) solution kept at 4 °C. The membranes were then placed within the chambers and cells seeded on them at a density of 0.3×10^4 cells/cm^2. The cells were then placed in a 37 °C cell culture incubator and allowed to adhere for 4 h. At the end of the static-culture period, the cells were gently washed with fresh medium to remove unattached cells or debris. The transepithelial electrical resistance (TEER) is measured using a Ag/Ag-Cl electrode (EVOM, World Precision Instruments) that applies an ac square wave current of +/− 20 μA amplitude at 12.5 Hz. The medium in the top chamber is then removed and the top access panel is closed. The medium flow is initiated at 1 μL/min followed by the introduction of air flow. The TEER measurements are taken at 24 h intervals excluding the pre- and post- pressure application readings.

For the dexamethasone trials, the cells were treated with 0.1μM dexamethasone prior to seeding the culture chamber. The following experimental steps are as detailed above.

D. Cell Viability Assay

In order to ascertain whether changes in TEER from pressure application are a result of drop in cell viability or a disruption of the cell layer integrity, we decided to perform the live-dead cell assay. The cells were stained with Calcein AM and Ethidium homodimer-1 (4mM Calcein AM in DMSO at 1:2000 dilutions, Molecular Probes L3224) by incubating cells in serum-free medium with the above reagents for an hour at room temperature. The cells were centrifuged at 1000rpm for 5 min and resuspended in their respective culture medium for seeding. Subsequent fluorescent images show viable cells as stained green while dead cells as stained red. Images were acquired prior- and post- pressure application.

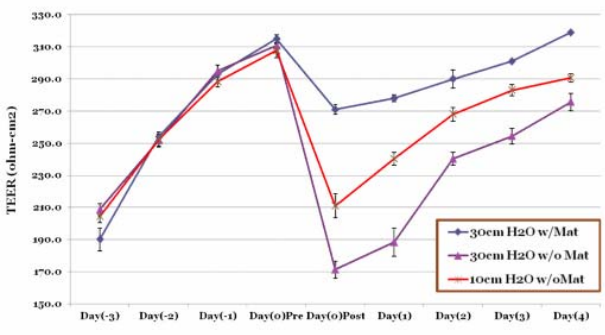
III. Results and Discussion

From our previous experiments we observed that alveolar cells in air-exposed cultures exhibited higher TEER values than the conventional submerged cultures. The air–exposed cultures also demonstrated lower surface tension measurements [4].

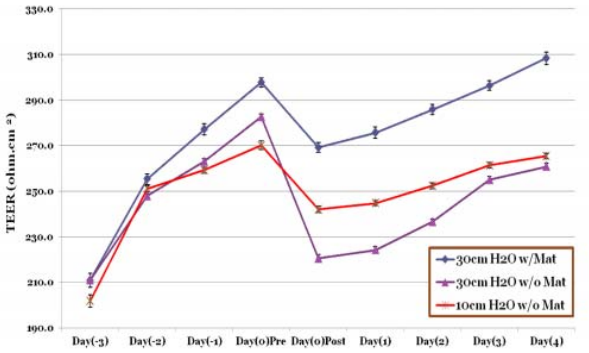
In line with these results, the air-exposed cultures exhibit gradual increase in TEER values for the first four days in culture from Day(-3) to Day(0). Day(0) corresponds to the day of pressure application followed by post-pressure period from Day(1) to Day(4). We observed that the TEER measurements taken post-pressure application were starkly lower than the pre-pressure values. This drop in TEER varied between cell types and cell treatments. We noticed that the A549 cells were more responsive to the added pressure stimuli by exhibiting a greater drop in TEER compared to the H441 cells [Fig. 2]. Negative controls were provided by air-exposed transwell cultures with similar membrane treatment (data not shown).

Cells cultured with an underlying matrigel-coating, showed lower drop in TEER in comparison to the untreated samples. We noted the cells with no coating to exhibit similar TEER values prior to pressure application but a greater TEER drop after exposure, continued by a weaker recovery. A possible explanation for the lower response curves in the matrigel-coated trials could be that it provides a buffering action to the compressive forces during pressure application and could also be providing the cells with growth factors that could aid formation of tighter alveolar cell layers with greater integrity. The mixture of extracellular matrix proteins in matrigel could also support maintenance of the predominantly type II alveolar cell phenotype, that is responsible of making surfactant proteins in response to air-exposure [5].

Additionally, dexamethasone treatment appears to mitigate the TEER drop since we observed lower percent drop in treated cells compared to the untreated samples (Fig. 3(a)). Simultaneously, dexamethasone treated samples appeared to follow shorter recuperation time than untreated cultures. After the post-exposure drop, the TEER values continue to rise reaching pre-exposure plateau values.



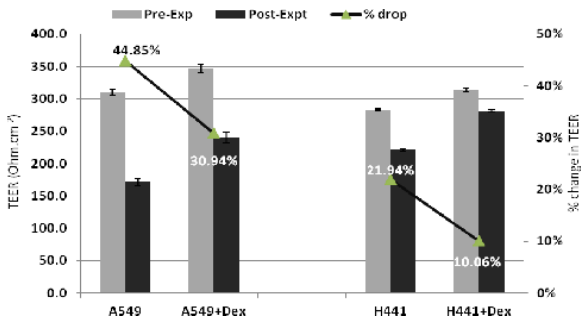
(a)



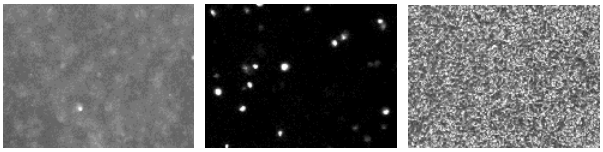
(b)

Fig. 2 TEER measurements taken during the duration of the experiment in 24h intervals. Comparison of samples with matrigel-coated and no-coating (a) A549 and (b) H441 cells

In regular culture the addition of 0.1 µM dexamethasone delayed cell proliferation in both the A549 and H441 cultures. However, once the cells reached confluence they exhibit considerable higher TEER readings compared to untreated cultures. This could indicate the formation of stronger tight junctions between the cells. Glucocorticoids with their anti-inflammatory properties could be mitigating the response of the alveolar cells in response to the pressure application.



(a)



(b) (c) (d)

Fig. 3 (a) Percentage change in TEER values between the pre- and post- pressure application measurements. Corresponding cell viability assay images taken post-exposure: A549 cells imaged under different filters (b) green fluorescence, (c) red fluorescence and (d) phase contrast image

The fluorescent images of the cells treated to the live-dead cell assay did not indicate substantial difference in the composition of the live-to-dead cells in the alveolar layer. Although there were a small percentage of dead cells in each trial, the composition did not change upon application of pressure. Not all of the excess cell debris is washed away during the single wash step after the static culture, leaving dead cells adhered to cell layer surface. Additionally, with the deep culture wells and 0.3 µm pore size membrane for culture surface, the cells have the propensity to grow out in small islands of cells intermittently on the monolayer. These pockets contain a greater concentration of dead cells due to the suffocation of cells unable to reach the medium supplemented from the lower chamber.

IV. Conclusion

The drop in TEER for the alveolar cells indicates a breach of their cell layer integrity from the additional air pressure. The live-dead cell assay helped assess that this effect is not strongly due to cell death. Hence it appears to be predominantly from the mechanical disruption of the cellular tight junctions. These results could be further investigated to determine composition of the cell layer between type I and type II alveolar epithelial cells to see whether the cells dedifferentiate in response to the pressure stimuli or the addition of dexamethasone. With the administration of antenatal and postnatal steroids in the treatment of underdeveloped lungs in premature infants, it would be beneficial to know their effect on alveolar cells and their ability to respond to altering pressure during mechanical ventilation.

Acknowledgment

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